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That I am knowledgeable in the English language and in the language in which the below identified application was filed, and that I believe the English translation of International Application No. PCT/JP00/06222 is a true and complete translation of the above identified International Application as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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SPECIFICATION

METHOD FOR PRODUCING MALE-STERILE PLANT

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The present application claims priority from Japanese Patent Application Hei 11-279307 filed on September 30, 1999, the disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to a method for producing a male-sterile plant by gene manipulation.

10 PRIOR ART

Barnase is an RNase derived from *Bacillus amyloliquefaciens* (S. Nishimura and M. Nomura, Biochem. Biophys. Acta 30, 430-431:1958; R.W. Hartley, J. Mol. Biol., 202, 913-915:1988). It is an enzyme having 110 amino acid residues and hydrolyzing RNA. When expressed in cells, this enzyme inhibits the functions of the cells owing to its potent RNase activity and thus causes cell death in many cases. By using this characteristic, it is therefore expected that expressing the barnase gene in a specific site of a plant would result in the selective regulation of the function of that site.

PCT WO89/10396 discloses a technique whereby a male-sterile plant is obtained by constructing a male sterility gene by ligating the above-described barnase gene to the downstream of an anther tapetal cell-specific expression promoter and transferring the thus obtained gene into a plant. This male sterilization technique is highly useful in efficiently developing an F1 hybrid variety.

When a barnase gene is employed as a male sterility gene, however, it is frequently observed that the thus obtained male- sterile transgenic plant exhibits unfavorable characteristics. PCT WO96/26283 refers to this problem in rice. It is also reported that similar phenomena are observed in lettuce (*Scientia Horticulturae* 55, 125-139:1993; Arlette Reymaerts, Hilde Van de Wiele, Greta De Sutter, Jan Janssens: Engineered genes for fertility control and their application in hybrid seed production). According to this report, a plant with depressed activity was constructed by transferring a male sterility gene comprising a tobacco-origin anther-specific promoter (TA29) and barnase into lettuce.

Although the causes of these phenomena have not been accurately determined so far, it is assumed that the so-called "position effect" of the gene transfer site might affect the mechanism (PCT WO96/26283). More specifically, the aimed male-sterile plant can be constructed when the male sterility gene is expressed exclusively in the target site (i.e., anthers). However, there is a possibility that the barnase might be expressed, even though in an extremely small amount, in tissues other than anthers under the action of an expression regulator (for example, an endogenous enhancer) existing in the vicinity of the gene transfer site. In such a case, the unfavorable characteristics as described above might arise due to the potent enzymatic activity.

To overcome this problem, PCT WO96/26283 discloses a

method using cauliflower mosaic virus 35S promoter
(hereinafter referred to as CaMV35S promoter) which is
expressed potently in tissues other than anthers. Namely,
a Barstar, i.e., an inhibitory protein against barnase is
5 employed therein. The Barstar gene ligated to the CaMV35S
promoter is transferred into a plant simultaneously and
then the Barstar gene is constitutively expressed in
tissues other than anthers, thereby eliminating the effects
of the barnase on tissues other than anthers.

10 In the male-sterile plant constructed by this method,
however, the Barstar gene is expressed in the whole plant
including leaves and albumen that is an edible part. It is
not desirable from the viewpoint of the public acceptance
of transgenic crops that the Barstar protein is expressed
15 in parts where its expression is unnecessary.

SUMMARY OF THE INVENTION

The present invention provides a method for producing
a male-sterile plant that has normal morphology comparable
to the intact species but being male-sterile.

20 The present invention provides a method for producing
a male-sterile plant by expressing a foreign RNase in the
anthers of the plant wherein the expression of the foreign
RNase in tissues other than anthers is inhibited as much as
possible to thereby construct a male-sterile plant showing
25 normal morphology comparable to the original (i.e., intact)
plant.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a model view which shows the structure of

plasmid pTS346 for transforming plant cells which contains
a Barstar gene under the control of an anther-specific E1
promoter and a barnase gene under the control of an anther-
specific E1 promoter fragment in order from the 5'-end to
5 the 3'-end.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors obtained two male sterile lines
showing improvement in that they respectively exhibited a
smaller degree of morphological abnormality from among rice
10 male-sterile lines having been transfected with a genetic
construct prepared by ligating a barnase gene to an anther-
specific E1 promoter originating in rice. By analyzing
these lines, it was found that the E1 promoter had been
divided into two parts by an about 10 kb transposon
15 originating in *Agrobacterium tumefaciens* LBA4404 employed
in the transformation of rice. Based on this fact, it was
assumed that the effect of the upper part of the E1
promoter on the expression of the barnase gene would have
been compromised by the intervention of the transposon of
20 10 kb or more and, as a result, the expression of the
barnase gene would have been depressed giving rise to the
alleviation of morphological abnormality.

This finding indicated that the problem of the
expression of an RNase in tissues other than anthers
25 encountered in the conventional art would be overcome if an
RNase gene (for example, a barnase gene) is ligated to the
downstream of an anther-specific promoter which is made
shorter than the full length (i.e., a promoter fragment)

and the resultant construct is transferred into a plant genome. From this viewpoint, the present inventors further conducted intensive studies and thus produced a transgenic rice plant by transferring a Barstar gene ligated to the downstream of a rice anther-specific E1 promoter simultaneously with an RNase gene ligated to a fragment of an anther-specific promoter fragment, thereby achieved further improvement in the morphology of male-sterile rice lines.

Accordingly, the present invention provides a method for producing a male-sterile plant characterized by comprising ligating a first promoter fragment to the upstream of an RNase gene, further ligating a second promoter, which is the same as the first promoter or different therefrom, to the upstream of an RNase inhibitor protein gene, and transferring these genes into a plant genome to thereby make the plant substantially male-sterile.

It is preferable that the RNase gene to be used in the present invention is barnase gene. Barnase gene may be employed as such. Alternatively, it may be naturally or artificially mutated so as to modify the activity of the enzyme encoded thereby or the tissue specificity thereof. For the purpose of the present invention, use can be made of the gene of any enzyme which can act as an RNase in plant cells (in particular, anthers) and thus inhibit protein biosynthesis in the cells. Examples of such enzymes include pancreas RNase A, RNase T1 from *Aspergillus oryzae* and Sarnase originating in *Streptomyces aureofaciens*

(European Patent Publication No. 053799).

5 The promoter to be ligated to the upstream of the
RNase gene (i.e., the first promoter) can be any promoter,
which can induce the expression of the RNase gene to be
employed in plant cells. As examples, tobacco TA29
promoter (J. Seurinck et al., Nuc. Acid. Res., 18, 3403,
1990) and *Arabidopsis* A9 promoter (Wyatt Paul et al., Plant
Mol. Biol., 19, 611-622, 1992) may be mentioned. It is
preferable that the first promoter is a promoter specific
10 to the anther of the plant which is to be made male-sterile.
In the case of rice, there are known anther-specific
promoters such as E1 promoter, T72 promoter, T42 promoter
(each described in PCT WO92/13956), Osg6B promoter, Osg4B
promoter (each reported by T. Tsuchiya et al., Plant Mol.
15 Biol. 26, 1737-1746, 1994) and the like. The sequence of
the E1 promoter is represented by SEQ ID NO:6.

It is essential to use the first promoter as a
fragment. The term "fragment" as used herein in connection
with the first promoter means a sequence that at least
20 partly differs from the promoter sequence occurring in
nature. For example, the fragment is a promoter having
deletion, substitution or insertion in the 3'-region, the
internal region and/or the 5'-region including a case of
being divided into upstream and downstream regions with an
25 intervening sequence. It is preferable that, as a result
of the deletion, substitution or insertion, the fragment
has up to about 50% homology, still preferably up to about
70% homology and particularly preferably up to about 90%

homology, with the natural promoter sequence. As one of the preferable examples of the first promoter fragments, the fragment originating in the E1 promoter as represented by SEQ ID NO:7 may be mentioned. Also, it is preferable to use, as the first promoter fragment, a sequence obtained by modifying the above-described sequence by substitution, deletion or insertion of one or more nucleotides, so long as it has the same promoter activity as the fragment represented by SEQ ID NO:7. The expression "to modify by substitution, deletion or insertion of nucleotides" as used herein means such a modification as is available by subjecting, for example, ten or less (preferably five or less) nucleotides to artificial manipulation such as site-specific mutagenesis.

The first promoter fragment is ligated to the upstream of the RNase gene in a functional manner. The term "functional" means that the promoter fragment is ligated to such a position as allowing inducing the expression of the RNase gene.

It is also essential in the present invention to use an RNase inhibitor protein gene. This gene may be appropriately selected depending on the RNase gene employed. In the case where the RNase gene is barnase gene, for example, it is preferable to use Barstar gene as the RNase inhibitor protein gene. A second promoter is ligated in a functional manner to the upstream of the RNase inhibitor protein gene. The second promoter, which induces the expression of the RNase inhibitor protein gene to be

employed in plant cells, may be either the same as the first promoter or different therefrom. In case where the second promoter is the same as the first promoter, it is not always necessary to employ the second promoter in the form of a fragment.

The RNase gene, which has been ligated to the first promoter fragment in a functional manner, may be located either downstream or upstream of the RNase inhibitor protein gene which has been ligated to the second promoter in a functional manner. Also, the distance between these genes is not particularly limited.

The plant that can be made male-sterile by the method of the present invention is not particularly limited. As examples thereof, rice, corn, tobacco, lettuce and rapeseed may be mentioned. Among all, rice and corn are particularly preferable.

To transfer the genes into a plant genome to make the plant male-sterile in the method of the present invention, use may be made of the Agrobacterium method, the electroporation method, the particle gun method, etc. It is preferable to use the Agrobacterium method. Details of the procedure may be appropriately determined by a person skilled in the art by reference to, for example, the method described in PCT WO92/13957. For example, the genes can be transferred into a plant cell genome by the following steps:

- (1) providing a first promoter fragment;
- (2) providing an RNase gene;

(3) ligating the RNase gene to the downstream of the first promoter fragment in a functional manner;

(4) providing a second promoter;

(5) providing an RNase inhibitor protein gene;

5 (6) ligating the RNase inhibitor protein gene to the downstream of the second promoter in a functional manner;

(7) integrating the RNase gene, which has been ligated to the downstream of the first promoter fragment, and the RNase inhibitor protein gene, which has been
10 ligated to the downstream of the second promoter, into T-DNA with the use of restriction enzyme sites;

(8) inserting the T-DNA from step (7) into a Ti plasmid;

(9) amplifying the Ti plasmid from step (8) in an
15 agrobacterium host cells, if needed; and

(10) infecting plant cells with the agrobacterium from step (9) in order to transfer the RNase gene, which has been ligated to the downstream of the first promoter fragment, and the RNase inhibitor protein gene, which has
20 been ligated to the downstream of the second promoter, into the genome of the plant cells.

The transgenic plant cells thus obtained can be regenerated into an individual plant starting with callus culture by using the method described in, for example, Y.
25 Hiei et al., Plant J. 6, 271-282:1994.

In the above-described process, the first promoter fragment may be prepared by cleaving the first promoter with appropriate restriction enzymes. Alternatively, it

may be prepared by amplifying the sequence, which is obtained by deleting unnecessary regions from a plasmid containing the first promoter and the RNase gene, by PCR with the use of appropriate primers, as will be described in Example hereinafter. Also, it is to be understood that various changes in the order of the above steps (in particular, steps (1) to (7)) or materials employed therein fall within the scope of the present invention. Further, it is possible to transform plant cells by using Ti plasmids containing different T-DNAs, of which one carrying the RNase gene which has been ligated to the downstream of the first promoter fragment, while the other carrying the RNase inhibitor protein gene which has been ligated to the downstream of the second promoter. Furthermore, calluses having the transferred genes can be easily selected by preliminarily providing a selection marker in the T-DNA of the step (7). Examples of such a marker include Bar gene employed in the following Example and a hygromycin resistant (HPT) gene. However, it is not always necessary to use a selection marker. The achievement of transformation may be judged based on the fact that the plants regenerated from calluses of transgenic cells have been made male-sterile.

The present invention further provides a vector for transferring the above genes into a plant cell genome by the agrobacterium method as described above. This is a vector which has a T-DNA containing i) a promoter fragment and an RNase gene expressible under the control of said

promoter, and ii) a promoter being the same as the above-described promoter or different therefrom and an RNase inhibitor protein gene expressible under the control of said second promoter, so that the vector can transfer the

5 T-DNA into a plant cell genome when placed in an agrobacterium-infected plant cells. It is preferable that the vector of the present invention is constructed by integrating the T-DNA containing the above-described genetic elements i) and ii) into Ti plasmid for

10 transforming plants. Various Ti plasmids for transforming plants are known and available at present. For example, pAL4404 carried by *Agrobacterium tumefaciens* strain LBA4404 can be mentioned. The vector according to the invention may contain, if necessary, one or more elements selected

15 from among a replication origin for amplification in an agrobacterium, terminators located respectively downstream of the RNase gene and the RNase inhibitor protein gene and/or a marker gene appropriate for selecting transgenic plant cells.

20 The present invention furthermore provides a transgenic plant cell which contains i) a promoter fragment and an RNase gene expressible under the control of said promoter, and ii) a promoter being the same as the above-described promoter or different therefrom and an RNase

25 inhibitor protein gene expressible under the control of said promoter, having been transferred into the genome, and a male-sterile plant regenerated from said transgenic cell.

EFFECT OF THE INVENTION

the original plasmid pTS172 was substituted by the 356 bp fragment represented by SEQ ID NO:7.

Next, a fragment (SEQ ID NO:4) comprising an E1 promoter (PCT WO92/13956) ligated to a Barstar gene (R.W. Hartley, J. Mol. Biol. 202, 913-915:1988) was blunt-ended and integrated into the PstI site of pTS172 Δ to give a plasmid pTS346 (SEQ ID NO:5). Fig. 1 illustrates the structure of pTS346.

A 5.5kb fragment was excised from pTS346 with EcoRI and then inserted into the EcoRI site of pSB11BS (the structure of which will be described just below). Further, the T-DNA region was integrated into an acceptor vector pSB1 (T. Komari et al., Plant J. 10, 165-174:1996) by way of homologous recombination. The resulted recombinant plasmid (pSB1346) was introduced into *Agrobacterium tumefaciens* LBA4404, which was used in transforming rice (variety: Asanohikari). The plasmid pSB11BS was constructed by integrating the Barstar gene (R.W. Hartley, J. Mol. Biol. 202, 913-915:1988) into the StuI site of intermediate vector pSB11 (T. Komari, Plant J. 10, 165-174:1996).

By this *Agrobacterium tumefaciens* LBA4404 having the recombinant plasmid, rice (variety: Asanohikari) was transformed. Although the transformation was performed basically in accordance with the method of Hiei et al. (Plant J. 6, 271-282:1994), phosphinothricin (concentration: 10 mg/L) was employed for screening transgenic plants since the male sterility-gene construct

contained a bar gene encoding phosphinothricin acetyl transferase as a selection marker.

A comparison with the construct pSB1172 (refer to PCT/JP99/04167 filed on August 3, 1999) comprising an E1 promoter of the normal length but lacking any Barstar gene indicated that the transformation efficiency and the ratio of transgenic plants with normal morphology were remarkably improved as shown in the following table.

10 Table 1: Transformation efficiency

	No. of infected calluses	No. of re-differentiated calluses	No. of PCR-positive lines	No. of morphologically normal male sterile lines
pSB1346	681	45	37/38*	15/37 (40.5%)
pSB1172 (control)	2838	83	52/83	9/52(17.3%)

*: examined 38 lines among 45.